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REMARKS

Claims 11-15, 21 and 22 are pending. No claim has been allowed.

Applicants gratefully acknowledge the withdrawal of the outstanding objections to the title and the specification of the instant application as well as the withdrawal of the rejection of claim 15 under 35 U.S.C. § 103 (a).

Rejection Under 35 U.S.C. §§ 101 and 112, first paragraph

Claims 11-14, 21 and 22 remain rejected under 35 U.S.C. §§ 101 and 112, first paragraph for reasons of record. The Examiner maintains that mRNA expression does not sufficiently correlate with protein expression to support the utility of a binding compound to the protein encoded by the disclosed novel nucleic acid sequence, relying primarily on the Haynes review to support her position. According to the Examiner, the subsequently published Gygi reference offers a positive correlation between mRNA expression and protein abundance with very highly expressed mRNAs, but not all mRNAs. Applicants traverse this rejection.

Applicants respectfully submit that the instant application fulfills the utility requirement as set forth in 35 U.S.C. § 101 because the mRNA data provided in the specification and in the Declaration of Jeanine Mattson pursuant to 37 C.F.R. § 1.132 demonstrates a specific and asserted utility. A copy of Dr. Mattson's declaration as submitted on April 28, 2003 is included herewith for the convenience of the Examiner. Quite simply, the Examiner maintains a position that the mRNA expression data presented in the specification as filed and in Dr. Mattson's declaration is insufficient to support a specific and substantial utility because she does not believe that the mRNA levels are sufficiently high to indicate that the protein encoded by the disclosed nucleic acid sequence, i.e., the RANK-like protein, is elevated and such elevation is detectable and correlative with a disease or disorder. Applicants submit that the standard applied by the Examiner cannot find support in the Haynes review or Gygi and is rebutted in its entirety by the data presented in Dr. Mattson's declaration.

First, the specification specifically identifies a utility that is supported by the disclosed data and Dr. Mattson's declaration. The specification states that the RANK-like protein in the

regulation and development of lymphocytes, and thus diseases associated with lymphocyte regulation and development. *See, e.g.*, the instant specification at page 57, lines 22-24. The specification then discloses the expression of the mRNA in art-recognized models of lymphocyte mediated response/disease, *i.e.*, allergic lung response. *See id.* at pages 30-31. This specifically asserted utility is further supported by Dr. Mattson's declaration where data using quantitative PCR demonstrates the expression of RANK-like mRNA in a primate model from idiopathic pulmonary fibrosis, a lymphocyte mediated disease state. *See* Mattson Declaration at ¶3-5. Applicants note that, to date, the Examiner has provided no scientific rationale or even a specific comment to explain why the data provided in Dr. Mattson's Declaration is insufficient.

Second, one of ordinary skill in the art would find a specific and substantial utility in the data presented in the specification and Dr. Mattson's declaration. As an example, Applicants provide an example in a recently published, peer-reviewed article where the authors draw the identical conclusions regarding utility as do the Applicants after applying the same technique used in Dr. Mattson's declaration. *See* Exhibit B (hereinafter "Li"). More particularly, Li employ Taqman, a quantitative PCR analysis, detection to examine the expression of a cytokine in rat cortical tissue before and after a short duration of ischemia. Li normalized the RNA samples using a housekeeping gene to further validate the Taqman results. Upon the observation of a significant induction of IL-1β mRNA (5-fold induction), Li declares that this data suggests a potential role of the cytokine in ischemic brain tolerance. Likewise, Applicants validated their original observations disclosed in the specification using Taqman analysis. *See* Exhibit A at ¶3. Applicants determined RANK-like mRNA levels in control lung samples from humans and *C. macaque* as well as mRNA levels in human lungs with idiopathic pulmonary fibrosis and *C. macaque* lungs after challenge with an inflammation-inducing pathogen, *i.e., Ascaris*. *Id.* There was a significant induction in both the human idiopathic pulmonary fibrosis (>20-fold induction) and *C. macaque* allergic lung samples (20-fold induction). *Id.* at ¶¶4-5 and Table 1. Based on such findings, the ordinary artisan would believe that it is more likely than not true that the RANK-like protein plays a role in some lymphocyte mediated diseases based on the data from these three different art-recognized models.

Applicants submit that there is no requirement to provide evidence sufficient to establish that an asserted utility is true "beyond a reasonable doubt" or "as a matter of statistical certainty."

See M.P.E.P. § 2107.02 (VII). An applicant is only required to provide evidence if, when considered as a whole, leads the skilled artisan to conclude that the asserted utility is more likely than not true. *See* M.P.E.P. § 2107.03 (II). Applicants have met that burden in the specification as originally filed.

Finally, Applicants remind the Examiner that a cytokine does not have to be expressed at a high level to definitively alter and influence the microenvironment or even the entire organ system in which it is expressed. Therefore, there is no scientific basis to support the Examiner's assertion that the RANK-link mRNA expression must fall within the range of highly expressed mRNAs cited by Gygi to be biologically relevant. Moreover, the data disclosed in each of the instant specification and in Dr. Mattson's declaration demonstrate an elevation of RANK-like mRNA in lungs with immune-mediated diseases over that of normal lung tissue. This further supports the Applicant's assertion of protein expression as even Gygi shows that even the mRNAs with the lowest copy number are also expressed as proteins. *See* Gygi at page 1727, Figure 5. Thus, there is no scientific rationale that supports the Examiner's position that the expression of RANK-like mRNA provided does not adequately support the asserted utility. Applicants have provided evidence of RANK-like mRNA expression that is demonstrably higher than that in normal tissues (where it was undetectable in some experiments) in three different models of lymphocyte mediated diseases. As the ordinary artisan would consider such evidence more likely than not to indicate a role for this novel cytokine in these lymphocyte-mediated diseases, this evidence is sufficient to meet the utility requirement of 35 U.S.C. § 101.

In view of the above, Applicants submit that the basis of the rejection may be removed.

Rejection Under 35 U.S.C. § 102 (e)

Claims 11-14, 21 and 22 are rejected under 35 U.S.C. § 102 (e) as allegedly being anticipated by Goddard et al., U.S. Published Application 20030092044, effective filing date April 12, 1999 for reasons of record. Applicants traverse this rejection.

Applicants submit that Goddard is not a proper reference under 35 U.S.C. § 102 (e) for reasons of record. Again, Applicants have met their burden with regards to the utility requirement for both the protein and the nucleic acid of the RANK-like protein, and therefore, Applicants

maintain their traversal of the Examiner's refusal to properly award a priority date of at least September 11, 1998. As the priority date is properly at least September 11, 1998, Goddard is not a reference under 35 U.S.C. § 102 (e) available against the instant application.

In view of the above, Applicants submit that the basis of the rejection may be removed.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to Deposit Account No. 03-1952 referencing docket no. 140942000401. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

Dated: May 10, 2004

Respectfully submitted,

By _____
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sd-191502

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL"

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to: Assistant Commissioner for Patents, Washington, D.C. 20231, on April 28, 2003.

~~Marian L. Christopher~~ Tami M. Procopio

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Erin E. MURPHY, *et al.*

Serial No.: 09/840,795

Filing Date: April 23, 2001

For: MAMMALIAN GENES; RELATED REAGENTS

Examiner: Eileen B. O'Hara

Group Art Unit: 1646

COPY

DECLARATION OF JEANINE D. MATTSON
PURSUANT TO 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Jeanine D. Mattson, declare as follows:

1. I am a co-inventor of the above-referenced patent application, and am familiar with the contents thereof.

2. I have a Master of Arts in Cell and Molecular Biology in 1992 from San Francisco State University, awarded in 1992, and a Bachelor of Science in Biology from University of California, Riverside, awarded in 1984. I am currently a Senior Scientist at the DNAX Research Institute and have worked with DNA since 1986. I have expertise in DNA library construction, hybridization, Southern blot analysis, isolation of total and polyA RNA, and PCR. A full curriculum vitae is attached as Exhibit D.

3. I have conducted experiments demonstrating that SEQ ID NO:17 was expressed in the lung during inflammation-mediated responses and/or diseases, as disclosed in the instant specification. The experimental results are set forth in the following paragraphs 3-6.

3. *Gene expression analysis.* Real time PCR analysis was employed to quantify gene expression. Briefly, mRNA was isolated using conventional methodology. The Taqman® system (Roche Molecular Systems) was then employed to quantify gene expression using the sequence of SEQ ID NO:17 per manufacturer's instructions. The three C-terminal amino acids were not included in the SEQ ID NO. 17 used in the Taqman® analysis. Using ubiquitin mRNA as the standard (or baseline) activity, relative increases in FRET expression were determined between various samples. The expression profile for the truncated SEQ ID NO:17 was determined using mRNA preparations from human tonsil, lung, colon, thyroid, and skin, as well as *C. macaque* lungs. *C. macaques*, a primate model, represented a recognized animal model for human disease. Samples included normal and diseased tissue. Of significance, idiopathic pulmonary fibrosis was a disease resulting from alveolar inflammation that resulted in fibrosis and interstitial pneumonia. Similarly, an *Ascaris* challenge in a *C. macaque* also resulted in alveolar inflammation.

4. In Experiment #1, the expression of truncated SEQ ID NO:17 was increased exclusively in lung undergoing an inflammatory reaction or disease state. As shown in Table 1, detectable SEQ ID NO:17 expression was not observed in control human lung or control *C. macaque* lung. However, lung with idiopathic pulmonary fibrosis showed a greater than 20 fold increase in expression relative to the control lung. Likewise, in the *C. macaque* samples, lung samples taken 24 hours post-challenge with *Ascaris* had a greater than 20 fold increase in SEQ ID NO:17 expression.

5. Experiment #2 also showed the expression of truncated SEQ ID NO:17 was increased during an inflammatory reaction or disease state. *See* Table 2. As seen in the previous experiment, truncated SEQ ID NO:17 expression was increased 24 hours post-*Ascaris* challenge in the *C. macaque* lung.

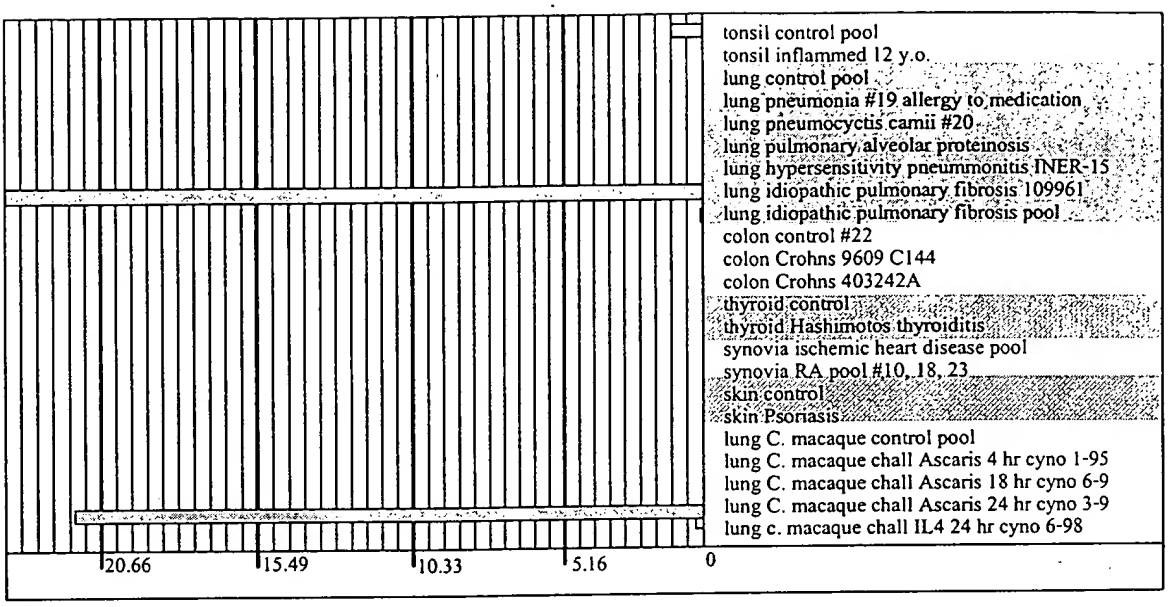
6. Taken together, these experiments demonstrated preferential expression of SEQ ID NO:17 in the lung during inflammation-mediated responses and/or diseases, as disclosed in the instant specification.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Executed at Palo Alto on April 25th, 2003.

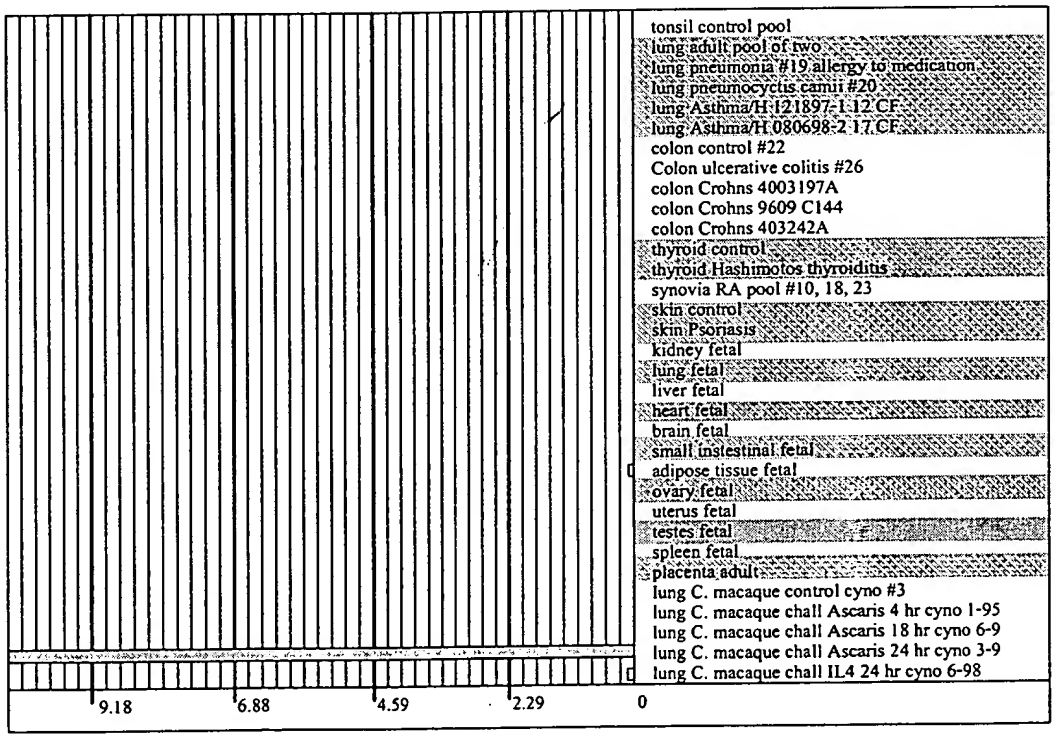
Jeanine D. Mattson

Table 1



Decrease Scale	Scale: Fold difference relative to Ubiquitin Sample Size:20.0 ng	Increase Scale

Table 2



Decrease Scale	Scale: Fold difference relative to Ubiquitin Sample Size:20.0 ng	Increase Scale

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Protocol

Application of real-time polymerase chain reaction for the quantitation of interleukin-1β mRNA upregulation in brain ischemic tolerance

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Accepted 4 February 2000

Abstract

Differential gene expression plays an important role in normal development and pathophysiological conditions. The accurate quantitation of mRNA expression is critical to assess the differential gene expression. While a number of techniques, such as Northern analysis, (semi-)quantitative reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization, are available to measure the levels of mRNA expression, certain limitations exist, including the insensitive and inaccurate quantitation of mRNA expressed at low abundance. In the present study, we describe the application of a recently developed TaqMan real-time quantitative RT-PCR for the detection of interleukin-1β (IL-1β) mRNA expression in rat cortical tissue after a short duration of ischemia (i.e., ischemic preconditioning). The principle of the TaqMan real-time detection is based on the fluorogenic 5′ nuclease assay that allows simple and rapid quantitation of a target sequence during the extension phase of PCR amplification. Using a cloned plasmid DNA as a standard and normalizing RNA samples with a housekeeping gene for the TaqMan real-time PCR, we detected the significant induction in absolute copy numbers of IL-1β mRNA in the ipsilateral cortex after preconditioning, suggesting a potential role of this inflammatory cytokine in ischemic brain tolerance.

Themes: Disorders of the nervous system

Topics: Ischemia

Keywords: Interleukin-1β; mRNA expression; Middle cerebral artery occlusion (MCAO); Ischemic preconditioning; Focal brain ischemia; TaqMan real-time quantitative RT-PCR

1. Type of research

- TaqMan real-time quantitative reverse transcription polymerase chain reaction (RT-PCR);
- Examining the expression of interleukin-1β (IL-1β) mRNA in ischemic brain tolerance.

2. Time required

- Generation of rat ischemic preconditioning samples (time course study) requires about a week.
- Preparation of total RNA from ipsilateral and contralateral cortices requires 4 h.
- Rat IL-1β and rpL32 cDNA cloning and amplification take 3 days.
- TaqMan probes and PCR primers designing and synthesis require about 1 day.
- Performing reverse transcription for all the preconditioning samples requires 1 h.
- Preparation of IL-1β and rpL32 plasmid serial dilution for standard curve requires 1 h.
- Preparation of PCR reaction and performing TaqMan PCR requires 4 h.
- Quantitative data analysis requires 0.5 h.

3. Materials

- Equipment
  - Facilities required for generating focal brain ischemia in rats [1]
  - Polytron (Brinkmann Instruments, Westbury, NY, USA)

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PII: S1385-299X(00)00015-5

- Table top microcentrifuge
- Speed-Vacuum
- Primer Express 1.0 Software program (PE Applied Biosystems, Foster City, CA, USA)
- GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems)
- ABI Prism 7700 Sequence Detection System (PE Applied Biosystems)
- Centrifuge with adapter for 96-well plate

- Supplies and reagents
  - TRIzol reagent (Gibco BRL, Rockville, MD, USA)
  - Chloroform
  - Isopropanol
  - Ethanol
  - RNase-free water
  - Rat IL-1β and rpL32 full-length cDNA plasmid clones [11]
  - Qiagen plasmid prep kit (Qiagen, Chatsworth, CA, USA)
  - MicroAmp Optical 96-Well Reaction Plate (PE Applied Biosystems)
  - MicroAmp Optical Tubes (PE Applied Biosystems)
  - MicroAmp Optical Eight-Strip Caps (PE Applied Biosystems)
  - TaqMan reverse transcription reagents: 100 U/μl of MultiScribe reverse transcriptase in 20 mM Tris–HCl, pH 7.5, 0.1 mM Na_2EDTA, 1 mM DTT, 0.01% (w/v) NP-40, and 50% (w/v) glycerol; 20 U/μl of RNase inhibitor in 20 mM HEPES–KOH, pH 7.6, 50 mM KCl, 8 mM DTT, and 50% glycerol; dNTP mixture (2.5 mM of each dATP/dCTP/dGTP/dTTP); 50 μM oligo $d(T)_{16}$ in 10 mM Tris–HCl, pH 8.3; 50 μM random hexamers in 10 mM Tris–HCl, pH 8.3; 10 × RT buffer containing 500 mM KCl, 100 mM Tris–HCl, pH 8.3; and 25 mM $MgCl_2$ solution (PE Applied Biosystems)
  - TaqMan PCR core reagents: 5 U/μl of AmpliTaq Gold DNA polymerase in 20 mM Tris–HCl, pH 9.0, 100 mM KCl, 0.1 mM EDTA, 1.0 mM DTT, 50% glycerol, and 0.5% (w/v) Tween 20; 1 U/μl of AmpErase UNG in 30 mM Tris–HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 5% glycerol, and 0.05% (w/v) Tween 20; 10 mM dATP/dCTP/dGTP; 20 mM dUTP; 10 × TaqMan buffer A in 500 mM KCl, 0.1 mM EDTA, 100 mM Tris–HCl, pH 8.3, and 600 nM passive reference; and 25 mM $MgCl_2$ solution (PE Applied Biosystems)
  - IL-1β forward primer: 5′-CACCTCTCAAGCAGAGCACAG-3′
  - IL-1β reverse primer: 5′-GGGTTCCATGGTGAAGTCAAC-3′
  - TaqMan probe for IL-1β: 5′-6FAM-TGTCCCGACCATTGCTGTTTCCTAGG-TAMRA-3′
  - rpL32 forward primer: 5′-TGTCCTCTAAGAACCGAAAAGCC-3′
  - rpL32 reverse primer: 5′-CGTTGGGATTGGTGACTCTGA-3′
  - TaqMan probe for rpL32: 5′-6FAM-TCGTAGAAAGAGCAGCACAGCTGGCC-TAMRA-3′
  - thin-walled PCR tubes
  - deionized or DEPC-treated water
  - TE buffer

4. Detailed procedures

4.1. Focal ischemic preconditioning

Transient middle cerebral artery occlusion (MCAO) (i.e., 10 min occlusion of MCA followed by reperfusion) or sham surgery was carried out in spontaneously hypertensive rats (SHR), 300–350 g, under sodium pentobarbital anesthesia as described previously [1]. Both ipsilateral and contralateral cortices were dissected from sham-operated rats (6 h) or following 1, 3, 6, 12 and 24 h, 2 and 5 days of a PC procedure. Each time point contains four rats ($n = 4$).

4.2. Total RNA isolation

Total cellular RNA was isolated from cortical samples using the TRIzol reagent according to manufacturer's description (Gibco BRL). Average RNA yield was approximately 1 μg RNA/mg rat brain tissue.

4.3. TaqMan probes and primers

PCR primers and TaqMan probes for IL-1β and rpL32 were designed using Primer Express 1.0 Software program (Perkin-Elmer). The rat IL-1β (accession number M98820) and rpL32 (accession number X06483) cDNA sequences were from GenBank. The TaqMan probes were labeled with a reporter fluorescent dye, FAM (6-carboxyfluorescein), at the 5′ end and a fluorescent dye quencher TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3′ end. The specificity of PCR primers was tested under normal PCR conditions in a thermal cycler prior to TaqMan PCR quantitation.

4.4. Reverse transcription

RT reactions were carried out for each RNA sample in MicroAmp reaction tubes using TaqMan reverse transcription reagents. Each reaction tube contained 3 μg of total RNA in a volume of 40 μl containing 1 × TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM of each dNTP, 2.5 μM of oligo-$d(T)_{16}$ primers, 0.4 U/μl of RNase inhibitor, and 1.25 U/μl of MultiScribe Reverse Transcriptase. RT reaction was carried out at 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. (Note: the incubation at 25°C for 10 min is necessary for the RT reaction with random hexamers or oligo $d(T)_{16}$ primers to obtain the optimal results; if

a sequence-specific primer is used, this incubation step is not necessary.) The RT reaction mixture was then placed at 4°C for immediate use of PCR amplification, or stored at −20°C for later use (similar results are expected at these two different temperatures of storage).

4.5. Construction of the IL-1β and rpL32 standard curve

To determine the absolute copy number of the target transcript, a cloned plasmid DNA for IL-1β and rpL32 was used to generate a standard curve. The plasmid DNA was purified using Qiagen maxi prep kit (Qiagen) according to the manufacture's directions. IL-1β and rpL32 plasmids contain 373 and 465 bp of cDNA inserts, respectively, in a pBluescript vector (2958 bp). The copy numbers of plasmid DNA template were calculated according to the molecular weight of the plasmid (6.6×10^5 kb^{-1} average value) and then converted into the copy numbers based upon the Avogadro's number (1 mol = 6.022×10^{23} molecules). The cloned plasmid DNA was serially (every fivefold) diluted at a range of 16.4 fg–2.56 pg (or 1.65–5.85 log molecules) and 2.05 pg–0.32 ng (or 3.74–7.93 log molecules) for IL-1β and rpL32, respectively. Log molecules equals log N, where N is the copy number of that molecule (e.g., 1000 molecules = 3 log molecules, or 1.65 log molecules of IL-1β = 45 copies of IL-1β). Each sample was run in triplicates, and the ΔR_n (the ratio of the amount of reporter dye emission to the quenching dye emission) and threshold cycle (C_t) values were averaged from each reaction.

4.6. TaqMan real-time quantitative PCR

The principle of the TaqMan real-time detection is based on the fluorogenic 5′ nuclease assay [4,6,8] as illustrated in Fig. 1. A thermal stable AmpliTaq Gold DNA polymerase was used for the PCR amplification. Real-time PCR was performed in a MicroAmp Optical 96-Well Reaction Plate. Each well contained 4 μl of each RT product (300 ng total RNA) or 4 μl of each plasmid DNA dilution, 1 × TaqMan buffer A, 5.5 mM $MgCl_2$, 200 μM dATP/dCTP/dGTP, 400 μM dUTP, 200 nM primers (forward and reverse), 100 nM TaqMan probe, 0.01 U/μl AmpErase, and 0.025 U/μl AmpliTaq Gold DNA polymerase in a total volume of 50 μl. Each well was closed with MicroAmp Optical caps (Perkin-Elmer), following complete loading of reagents. Amplification conditions were 2 min at 50°C (for AmpErase UNG incubation to remove any uracil incorporated into the cDNA), 10 min at 95°C (for AmpliTaq Gold activation), and then run for 40 cycles at 95°C for 15 s, 60°C for 1 min.

All reactions were performed in the ABI Prism 7700 Sequence Detection System for the test samples, standards, and no template controls were run in triplicates using the Sequence Detector V 1.6 program. The ΔR_n and C_t were averaged from the values obtained in each reaction. A "standard curve" was constructed by plotting the C_t vs. the known copy numbers of the template in the standard. According to the standard curve, the copy numbers for all unknown samples were obtained automatically.

4.7. Normalization of IL-1β mRNA levels to that of rpL32 in the cortical samples

The absolute copy number of IL-1β and rpL32 mRNA in each sample was calculated based on its C_t value with its plasmid DNA standard curve. The absolute copy num-

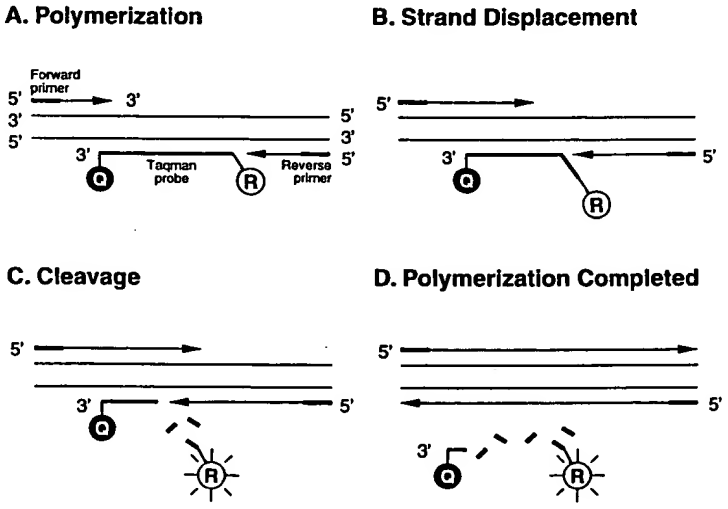

Fig. 1. A schematic illustration of 5′ nuclease assay for TaqMan real-time PCR. (A) A TaqMan probe labeled with a reporter fluorescent dye (FAM) at the 5′ end and a quencher-fluorescent dye (TAMRA) at the 3′ end hybridizes to the target cDNA. When the probe is intact, the reporter dye emission is quenched, owing to the physical proximity of the reporter (R) and quencher (Q) dyes. (B–D) During the polymerization chain extension, the 5′ nuclease activity of the DNA polymerase cleaves the hybridized probe and releases the reporter dye from the probe. A sequence detector can now detect the emission of the released reporter dye, and the relative signal increases in real-time during PCR amplification.

ber of IL-1β mRNA was then normalized to rpL32 to minimize variability in the results due to differences in the RT efficiency and RNA integrity among test samples.

4.8. Data analysis

Statistical comparisons were performed using ANOVA (Fisher's protected least squares difference). Values were considered to be significant when $p < 0.05$.

5. Results

Using a series of diluted plasmid DNA as templates, a standard curve for IL-1β and rpL32 was obtained using TaqMan real-time PCR (Fig. 2). Since the C_t value decreases linearly with the increasing amount of IL-1β plasmid DNA copy number and all of our tested samples were located within this linear amplification range, the copy number of IL-1β transcript in our test samples could be measured by using the standard curve.

Because the measurement of RNA concentration is often inaccurate, the difference among RNA samples is usually corrected using a housekeeping gene that is consistently expressed in the particular tissue. In the present study, rpL32 was selected as the internal control housekeeping gene because it was consistently expressed in cortical samples after PC [14] and focal stroke [13]. The levels of rpL32 mRNA expression in the cortical samples are illustrated in Fig. 3B, showing $1.11-1.53 \times 10^5$ copies of transcript per nanogram total RNA (or μg tissue). The normalized quantitative data ($n = 4$) of IL-1β mRNA expression in cortical samples after PC are illustrated in Fig. 3A. Low levels of IL-1β mRNA (one to four copies of mRNA per nanogram total RNA per microgram tissue) were observed in the contralateral cortex. While the levels of IL-1β mRNA expression were elevated in the ispsilat-

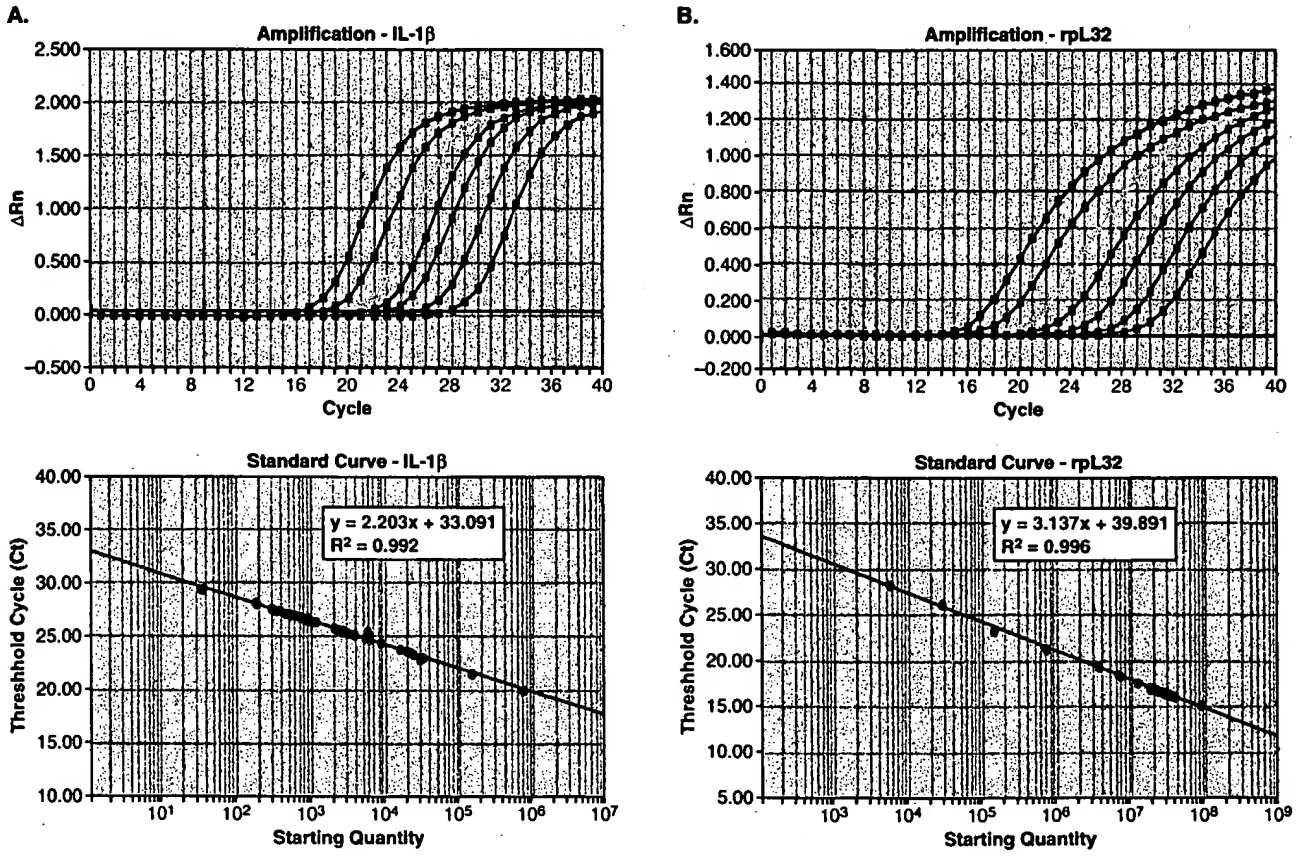

Fig. 2. Real-time PCR of IL-1β and rpL32 standard curves using a cloned plasmid DNA as a template. Amplification plots (upper panel) show the accumulation of IL-1β (A) and rpL32 (B) cDNA in real-time PCR detected by the ABI Prism 7700 Sequence Detector. Fivefold serial dilutions of the plasmid DNA were used as the templates, ranging 1.65–7.24 log molecules for IL-1β and 3.04–7.93 log molecules for rpL32 (log molecules = log N, where N is the copy number of that molecule). R_n (= the normalized reporter signal) represents the fluorescent signal of the reporter dye (i.e., the reporter dye emission) divided by the fluorescent signal of passive reference dye (i.e., the quenching dye emission). ΔR_n represents the normalized reporter signal minus the baseline signal established in the first few cycles of PCR. C_t (threshold cycle) represents the PCR cycle at which the reporter fluorescence above the baseline signal can be detected, i.e., the cycle number at which ΔR_n crosses the fixed threshold baseline. For each dilution of the standard, the ΔR_n is plotted against the cycle number. The lower panels plot the input DNA templates (log copy numbers) against the C_t values. All of our testing samples (reverse-transcribed from 300 ng of total cellular RNA) are within the standard range (10^2-10^5 molecules for IL-1β and 10^5-10^8 molecules for rpL32).

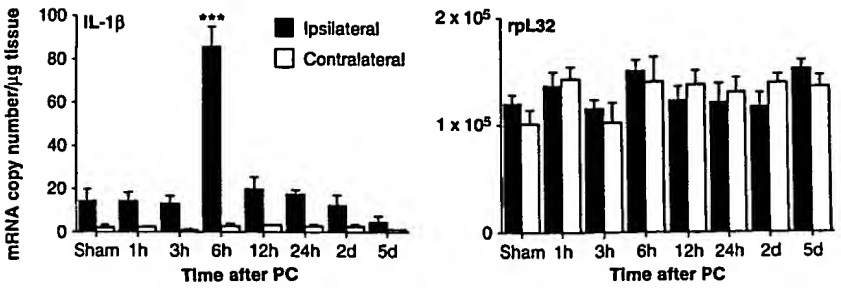

Fig. 3. Real-time PCR analysis of temporal expression of IL-1β mRNA in rat cortical samples after preconditioning. Total cellular RNA isolated from ipsilateral and contralateral cortices of rats subjected to sham surgery (6 h) or following 1, 3, 6, 12 and 24 h, and 2 and 5 days of a preconditioning procedure. RT-PCR was carried out using TaqMan real-time RT-PCR approach. Data were analyzed based on C_t values of each sample and normalized with rpL32. The absolute copy numbers of IL-1β were determined. Quantitative data ($n = 4$) for IL-1β mRNA expression after PC were displayed graphically (left). The rpL32 data ($n = 4$) were determined based upon the C_t value and reflected the unnormalized levels in each sample (right). ***$p < 0.001$, compared to sham-operated animals.

eral cortex after preconditioning and even in sham-operated samples, significant induction in IL-1β mRNA was observed only at 6 h after PC (87 copies of mRNA per microgram tissue, or fivefold increase compared to sham, $p < 0.001$).

6. Discussion

The use of TaqMan real-time PCR demonstrates the feasibility of this technique to quantitate the IL-1β mRNA transcript in rat brain ischemia after preconditioning. The levels of IL-1β mRNA expression in the cortex after preconditioning were not detectable by means of Northern analysis and were inconsistently detected by semi-quantitative RT-PCR [14]. This study clearly demonstrated the advantages of TaqMan real-time PCR over other existing methods for RNA quantitation, such as Northern blot analysis and (semi-)quantitative RT-PCR, not only on its sensitivity but also on its reduced carryover contamination, rapid, accurate and simultaneous quantitation of multiple sequential samples. Using a cloned plasmid DNA as a standard and a housekeeping gene (e.g., rpL32) to normalize the data, we are able to determine the absolute copy numbers of any particular mRNA using the real-time PCR. It also should be pointed out that this quantitation is based on the plasmid DNA as the template for the standard to measure the reverse-transcribed mRNA, assuming that their amplification rates are the same.

6.1. Troubleshooting

6.1.1. Location of PCR primers and TaqMan probes

It is important to have specific PCR primers and TaqMan probes for the real-time PCR. Therefore, it is recommended to have the primers and probes designed in 3′-untranslated region of the mRNA. The primers and probes are designed using Primer Express software to meet the specific criterion for real-time PCR.

6.1.2. Guidelines for primer designing

The primers are recommended to have a melting temperature (T_m) of 58–60°C for both forward and reverse primers. The primers can range at 15–30 bases in length and the G + C content at 30–70%. It is important to avoid runs of an identical nucleotide, especially for Gs, to ensure specific amplification. The total number of Gs and Cs in the last five nucleotides at the 3′ end of the primer should not exceed two. The optimum primer concentration must be determined empirically by testing concentrations in the 50–900 μM ranges. Primer sequences should not be self-complementary, or complementary to each other, particularly at the 3′ ends. It is also essential to place the forward and reverse primers as close as possible to the probe, but without overlapping the probe. The preferable amplicon size is smaller than 150 bp. The maximum amplicon size should not exceed 400 bp. Prior to TaqMan PCR, it is better to confirm the size and specificity of the PCR products by gel electrophoresis.

6.1.3. Guidelines for probe designing

The probe was designed with a T_m of 68–70°C. The estimated T_m for the probe should be 5–10°C higher than the estimated T_m for the primers. There is no G on the 5′-end of the probe. It is critical that the TaqMan probe is target-sequence-specific and the sequence can be chosen either from upper stand or the lower strand. The strand with more Cs than Gs is recommended to synthesize the TaqMan probe. It is preferable to use a range of probe concentration (50–200 nM) to maximize the florescent signal.

6.1.4. Construction of plasmid DNA standard curve

The plasmid DNA can be used as template to generate a standard curve. The plasmid DNA concentration is measured at absorption wavelength of 260 nm and converted to the copy numbers using the molecular weight of the DNA. It is important that the DNA is pure and only contains a single species. Plasmid DNA prepared from *Escherichia coli* is often contaminated with RNA, which

increases the value of A_{260} measurement and thus, inflates the copy number of the plasmid. In addition, accurate pipetting is required because the standard must be diluted over several orders of magnitude. Plasmid DNA must be concentrated in order to measure an accurate A_{260} value. The concentrated DNA needs to be diluted with TE buffer to 10^6–10^{12}-fold to reach a concentration similar to the target transcript in biological samples. The stability of the diluted standards must be considered, especially for RNA. The diluted standards should be divided into small aliquots, stored at −80°C, and thawed only once before use. The diluted standards are stable at −80°C for at least 1 year.

6.1.5. Minimization of PCR contamination

Because the interpretation of results depends upon the comparison of a sample that contains template to a no-template control, it is extremely important that all sources of contamination should be anticipated and controlled. All reaction mixtures should be set up in an area isolated from PCR product analysis and sample preparation. All amplified PCR products should never be brought into the PCR set-up area. During the dilutions and PCR preparation, it is important to carefully open and close all sample tubes, and keep reactions and components capped as much as possible.

6.2. Alternate and support protocols

The real-time PCR represents a novel technology based upon the 5′ nuclease activity of the Taq DNA polymerase. Early methods of quantitative PCR rely on setting up the PCR using a housekeeping gene as an internal control (such as the use of aldolase A, β-actin and rpL32) for co-amplification [2,3,12]. However, this technique is only semi-quantitative since the templates and primers are not identical. Later, competitive RT-PCR methods have been developed that applied the same PCR primers and the same templates, except for a small insertion or deletion in the templates or the same primers but totally different templates [5,9,10]. These methods have the disadvantage since amplification efficiency may contribute to the inaccuracy of the quantitation. Compared to all these methods, the real-time PCR uses the number of cycles needed to reach a threshold amount of PCR product as a measure of the initial concentration of the target nucleic acid [4,6,8]. Therefore, TaqMan PCR for quantitation of mRNA is sensitive, accurate and high throughput. Since the initial development of the real-time PCR, some modifications on this technique have been made. For example, glass microcapillaries were used for the detection of single starting genomic DNA template molecules [7], and the cloned plasmid DNA template and a house keeping gene were used for the measurement of the absolute copy numbers of the transcripts (the present work). The advantages of real-time PCR and its further modification will make this technique more attractive for the quantitation of differential gene expression in various biological systems.

7. Quick procedure

- Preparation of rat brain tissue samples following ischemic preconditioning.
- Isolation of total cellular RNA from ipsilateral and contralateral cortices using a standard protocol.
- Designing PCR primers and TaqMan probes using Primer Express 1.0 Software program from PE Applied Biosystems.
- Performing reverse transcription reactions with approximately 3.0 μg of total RNA.
- Preparation of serial plasmid DNA dilutions as standard samples.
- Performing TaqMan PCR in ABI Prism 7700 Sequence Detection System.
- Construction of standard curve by plotting the C_t vs. the known copy number of each standard sample.
- Quantitation of IL-1β absolute transcript (or other test genes) copy numbers from unknown samples according to the standard curve.
- Normalization of IL-1β mRNA expression to the rpL32 housekeeping gene.
- Data analysis.

8. Essential literature references

Essential references: Refs. [4,6,8,14].

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